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Esculetin attenuates receptor activator of nuclear factor kappa-B ligand-mediated osteoclast differentiation through c-Fos/nuclear factor of activated T-cells c1 signaling pathway

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ABSTRACT

Esculetin exerts various biological effects on anti-oxidation, anti-tumors, and anti-inflammation. However, the involvement of esculetin in the bone metabolism process, particularly osteoclast differentiation has not yet been investigated. In the present study, we first confirmed the inhibitory effect of esculetin on receptor activator of nuclear factor-κB ligand (RANKL)-induced osteoclast formation. We then revealed the relationship between esculetin and the expression of osteoclast-specific molecules to elucidate its underlying mechanisms. Esculetin interfered with the expression of c-Fos and nuclear factor of activated T cell c1 (NFATc1) both at the mRNA and protein level with no involvement in osteoclast-associated early signaling pathways, suppressing the expression of various transcription factors exclusively expressed in osteoclasts such as *tartrate-resistant acid phosphatase* (*Trap*), *osteoclast-associated receptor* (*Oscar*), *dendritic cell-specific transmembrane protein* (*Dcstamp*), *osteoclast stimulatory transmembrane protein* (*Ocstamp*), *cathepsin K*, *αvβ3 integrin*, and *calcitonin receptor* (*Ctr*). Additionally, esculetin inhibited the formation of filamentous actin (F-actin) ring-positive osteoclasts during osteoclast differentiation. However, the development of F-actin structures and subsequent bone resorbing activity of mature osteoclasts, which are observed in osteoclast/osteoblast co-culture systems were not affected by esculetin. Taken together, our results indicate for the first time that esculetin inhibits RANKL-mediated osteoclastogenesis *via* direct suppression of c-Fos and NFATc1 expression and exerts an inhibitory effect on actin ring formation during osteoclastogenesis.

1. Introduction

Osteoporosis has become one of the most common bone-related disorders with aging in the human society. One of the major pathogenetic mechanisms involved in osteoporosis is an abnormally high rate of osteoclast differentiation (responsible for bone resorption) compared to osteoblast differentiation (responsible for bone formation), leading to the loss of bone mineral density, progressive disruption of bone architecture, and increased fracture frequency [1]. This imbalance between osteoclastic bone resorption and osteoblastic bone formation causes the development of rheumatoid arthritis, periodontitis, and osteolytic malignancies [2–4]. Therefore, the identification of intracellular processes related to osteoclast formation and functional activity might provide solutions to manage various metabolic bone diseases.

During the differentiation of monocyte/macrophage lineage precursor cells into multinucleated osteoclasts, receptor activator of nuclear factor-κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) are two essential cytokines recognized as potent inducers of osteoclastogenesis [5,6]. RANKL, also termed tumor necrosis factor-related activation-induced cytokine

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(TRANCE), osteoclast differentiation factor (ODF), and osteoprotegerin (OPG) ligand (OPGL), is identified as a protein belonging to the tumor necrosis factor (TNF) superfamily. The interaction of RANKL with members of the TNF receptor (TNFR) superfamily, OPG and RANK, is a prerequisite step to induce the initiation of osteoclast differentiation [7,8]. A previous study revealed that *Rankl* genetic deletion in mice causes not only osteoclast dysfunction *in vitro*, but also osteopetrotic conditions and defective dental erosion *in vivo* [5]. M-CSF, also called colony-stimulating factor-1 (CSF-1), plays a critical role in the multiplication, survival, and motility of early osteoclast precursors. The binding of M-CSF to its cell surface receptor, colony-stimulating factor-1 receptor (c-FMS), generates the activation of intracellular mediators, including extracellular signal-regulated protein kinases (ERK)1/2 and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) signaling, triggering osteoclast differentiation [9]. It has been previously shown that *op*/*op* mice with a point mutation of CSF1 gene cannot express functional M-CSF, suffering from osteoclast-poor osteopetrosis. In addition, this abnormal condition is rescued by treatment with a soluble form of M-CSF [10,11]. In response to RANKL and M-CSF, bone marrow-derived macrophages (BMMs) are differentiated into bone resorbing osteoclasts *via* multiple downstream pathways such as the nuclear factor-κB (NF-κB), mitogen-activated protein kinases (MAPKs), and phospholipase C gamma 2 (PLCγ2)-Ca^{2+} signaling pathways and the subsequent nuclear translocation of two main transcription factors, c-Fos and nuclear factor of activated T cell c1 (NFATc1) [12–14].

Esculetin (6,7-dihydroxycoumarin) is a naturally occurring coumarin compound that is isolated from various plant species such as *Artemisia scoparia*, *Artemisia capillaries*, *Euphorbia lathyris*, and *Citrus limonia* [15,16]. Coumarins, containing fused benzene and α-pyrone rings, belong to a group of phenolic substances, which can influence their bioactivities through the type of substitutions in their basic rings. Previous studies showed that esculetin possesses multifarious pharmacological activities such as antioxidant, anti-inflammatory, and anti-tumor activities [17–19]. However, there has been no reported study investigating the biological properties of esculetin on bone metabolism, particularly osteoclast differentiation. Therefore, this study was designed to assess the influence of esculetin on RANKL-induced osteoclastogenesis and its underlying mechanisms as well as its effect on osteoclastic function containing filamentous actin (F-actin) ring formation and bone resorbing activity.

2. Materials and methods

2.1. Reagents

Esculetin was purchased from Sigma (St. Louis, MO, USA). Recombinant soluble human RANKL and M-CSF were obtained from PeproTech EC Ltd. (London, UK). Penicillin/streptomycin antibiotics, α-minimum essential medium (α-MEM), and fetal bovine serum (FBS) were bought from Gibco BRL (Grand Island, NY, USA). 1,25-dihydroxyvitamin D_3 ($VitD_3$) and prostaglandin E_2 (PGE_2) were purchased from Sigma. Anti-c-Fos, NFATc1, phospho-phospholipase C gamma (PLCγ2), and PLCγ2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho-p38, p38, phospho-ERK1/2, ERK1/2, phospho-c-jun N-terminal kinase (JNK), JNK, phospho-Akt, Akt, Bruton's tyrosine kinase (Btk), and phospho-inhibitory κB (IκB) antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-phospho-Btk was bought from GeneTex (Irvine, CA, USA). Monoclonal β-actin antibody was obtained from Sigma. All other chemicals were of analytical grade or complied with the standards needed for cell culture experiments.

2.2. Experimental animals

Five-week-old ICR male mice were purchased from Damul Science (Daejeon, Korea) and housed in controlled temperature (22–24 °C) and humidity (55–60%) with 12 h light/dark cycles. All experiments in this study were performed in accordance with the animal experiment guidelines of the Institute Committee of Wonkwang University (Permit number: WKU14-98).

2.3. Mouse BMMs preparation and osteoclast differentiation

Mouse BMMs were isolated and cultured as described previously [20].

2.4. Cell viability assay, western blot analysis, quantitative real-time reverse transcription (RT)-PCR analysis, and retroviral gene transfection

XTT assay, western blot analysis, quantitative real-time RT-PCR analysis, and retroviral gene transfection were performed as described previously [20]. Primers used for real-time RT-PCR are summarized in Table 1.

2.5. F-actin ring staining

BMMs were cultured for 3 days with M-CSF (30 ng/mL) and RANKL (100 ng/mL) in the presence or absence of esculetin. Next, the cells were fixed with PBS containing 3.7% formalin for 20 min and permeabilized with PBS containing 0.1% Triton-X-100 for 15 min. The cells were then blocked with 2.5% bovine serum albumin (BSA) for 30 min and stained with phalloidin (Molecular Probes, Eugene, OR, USA) and DAPI (Sigma) at room temperature for 30 min. The cells were then mounted with histochoice mounting media solution (Amresco, Solon, OH, USA). The slides were examined with a FV1000 confocal laser scanning microscope (Olympus, Tokyo, Japan). The fluorescence intensity was measured and analyzed using FluoView software ver.2.0 (Olympus). Primary osteoblasts and bone marrow cells (BMCs) were co-cultured on a collagen gel-coated culture dish in the presence of $VitD_3$ and PGE_2 for 7 days. The co-cultured cells were detached by treatment with 0.1% collagenase at 37 °C for 10 min. Separated cells were then

Table 1
Primer sequences used for real-time RT-PCR analysis.

Gene name		Primer sequence (5′–3′)
Gapdh	Forward	5′-TCA AGA AGG TGG TGA AGC AG-3′
	Reverse	5′-AGT GGG AGT TGC TGT TGA AGT-3′
c-Fos	Forward	5′-GGT GAA GAC CGT GTC AGG AG-3′
	Reverse	5′-TAT TCC GTT CCC TTC GGA TT-3′
Nfatc1	Forward	5′-GAG TAC ACC TTC CAG CAC CTT-3′
	Reverse	5′-TAT GAT GTC GGG GAA AGA GA-3′
Trap	Forward	5′-TCA TGG GTG GTG CTG CT-3′
	Reverse	5′-GCC CAC AGC CAC AAA TCT-3′
Oscar	Forward	5′-GGA ATG GTC CTC ATC TCC TT-3′
	Reverse	5′-TCC AGG CAG TCT CTT CAG TTT-3′
Dcstamp	Forward	5′-TCC TCC ATG AAC AAA CAG TTC CA-3′
	Reverse	5′-AGA CGT GGT TTA GGA ATG CAG CTC-3′
Ocstamp	Forward	5′-ATG AGG ACC ATC AGG GCA GCC ACG-3′
	Reverse	5′-GGA GAA GCT GGG TCA GTA GTT CGT-3′
Cathepsin K	Forward	5′-CCA GTG GGA GCT ATG GAA GA-3′
	Reverse	5′-CTC CAG GTT ATG GGC AGA GA-3′
integrin-αv	Forward	5′- ACA AGC TCA CTC CCA TCA CC-3′
	Reverse	5′- ATA TGA GCC TGC CGA CTG AC-3′
integrin-β3	Forward	5′-GGA GTG GCT GAT CCA GAT GT-3′
	Reverse	5′-TCT GAC CAT CTT CCC TGT CC-3′
Ctr	Forward	5′-TCC AAC AAG GTG CTT GGG AA-3′
	Reverse	5′-CTT GAA CTG CGT CCA CTG GC-3′

plated on 96-well plates with or without esculetin for 24 h. Cells were then stained with phalloidin and DAPI as described above and F-actin ring distribution was visualized using a fluorescence microscope (DMLB, Leica, Wetzlar, Germany).

2.6. Resorption pit assay

BMCs were co-cultured with primary osteoblasts using a similar method and replated on hydroxyapatite-coated plates with or without esculetin for 24 h. Next, the cells were removed and resorption pit areas were photographed and analyzed by the Image Pro-Plus program version 4.0 (Media Cybernetics; Silver Spring, MD, USA).

2.7. Statistical analysis

Each experiment was performed at least three times and all quantitative data are presented as mean ± standard deviation (SD). All statistical analyses were performed using SPSS software (Korean version 14.0; SPSS, Chicago, IL, USA). Student's *t*-test was

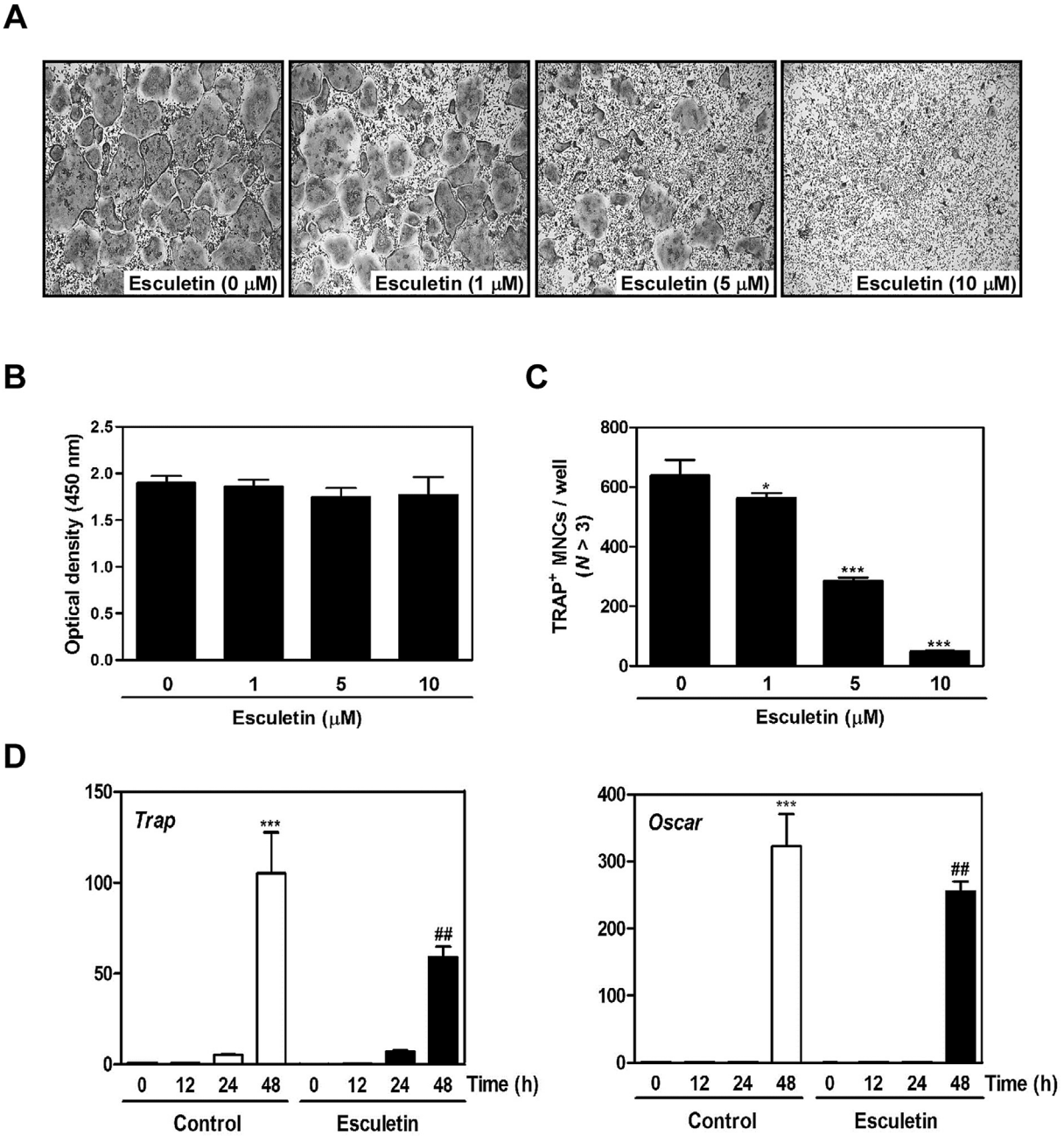

Fig. 1. Esculetin suppresses the formation of TRAP-positive osteoclasts without cytotoxicity (A) BMMs were cultured for 3 days with M-CSF (30 ng/mL) and RANKL (100 ng/mL) in the absence or presence of esculetin (10 μM). The cells were stained with TRAP solution. TRAP-positive cells were photographed under a light microscope. (B) BMMs were seeded into a 96-well plate and cultured for 3 days in the presence of M-CSF (30 ng/mL) with the indicated concentrations of esculetin. After 3 days, cell viability was analyzed by XTT assay. (C) TRAP-positive multinucleated cells ($TRAP^+$ MNCs) with more than three nuclei were counted as osteoclasts. $^{*}P < 0.05$, $^{***}P < 0.001$ *vs*. control group. (D) BMMs were pretreated with or without esculetin (10 μM) for 1 h in the presence of M-CSF (30 ng/mL) and then stimulated with RANKL (100 ng/mL) for the indicated times. The mRNA expression levels of *Trap* and *Oscar* were analyzed by quantitative real-time RT-PCR. $^{***}P < 0.001$ *vs*. control group at 0 h and $^{\#\#}P < 0.01$ *vs*. control group at the indicated time.

used to compare the parameters between 2 groups, while the analysis of variance (ANOVA) test, followed by the Tukey and Newman–Keuls post-hoc tests were used to compare the parameters among 3 groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Esculetin inhibits RANKL-induced osteoclast differentiation without any cytotoxic effects

In order to investigate the effect of esculetin on osteoclast differentiation, we cultured mouse primary BMMs treated with M-CSF and RANKL in the presence or absence of different concentrations of esculetin. The number of TRAP-positive osteoclasts increased in the control group, while esculetin inhibited the formation of TRAP-positive osteoclasts in a dose dependent-manner (Fig. 1A and C). This esculetin inhibitory effect on osteoclastogenesis did not affect cell viability (Fig. 1B). Next, in order to estimate the effects of esculetin on the expression of *Trap* and *Oscar*, we performed quantitative real-time RT-PCR. *Trap* and *Oscar* mRNA expression levels were suppressed by esculetin (Fig. 1D).

3.2. Esculetin regulates osteoclast differentiation without alteration of early signaling pathways

To define the molecular mechanisms underlying the inhibitory effect of esculetin on osteoclastogenesis, BMMs were treated with esculetin at three different time points in the presence of M-CSF and RANKL. The results showed that esculetin significantly inhibited osteoclast differentiation when the cells were exposed to esculetin on day 0–1 and 1–2 after RANKL treatment, but not on day 2–3 (Fig. 2A). However, esculetin did not affect any early signaling pathways-mediated by RANKL, including the JNK, Akt, p38, ERK, IκB, PLCγ2, and Btk pathways (Fig. 2B).

3.3. Esculetin inhibits RANKL-induced expression of c-Fos and NFATc1

To examine whether esculetin regulates RANKL-mediated osteoclastogenesis by downregulating c-Fos and NFATc1, we

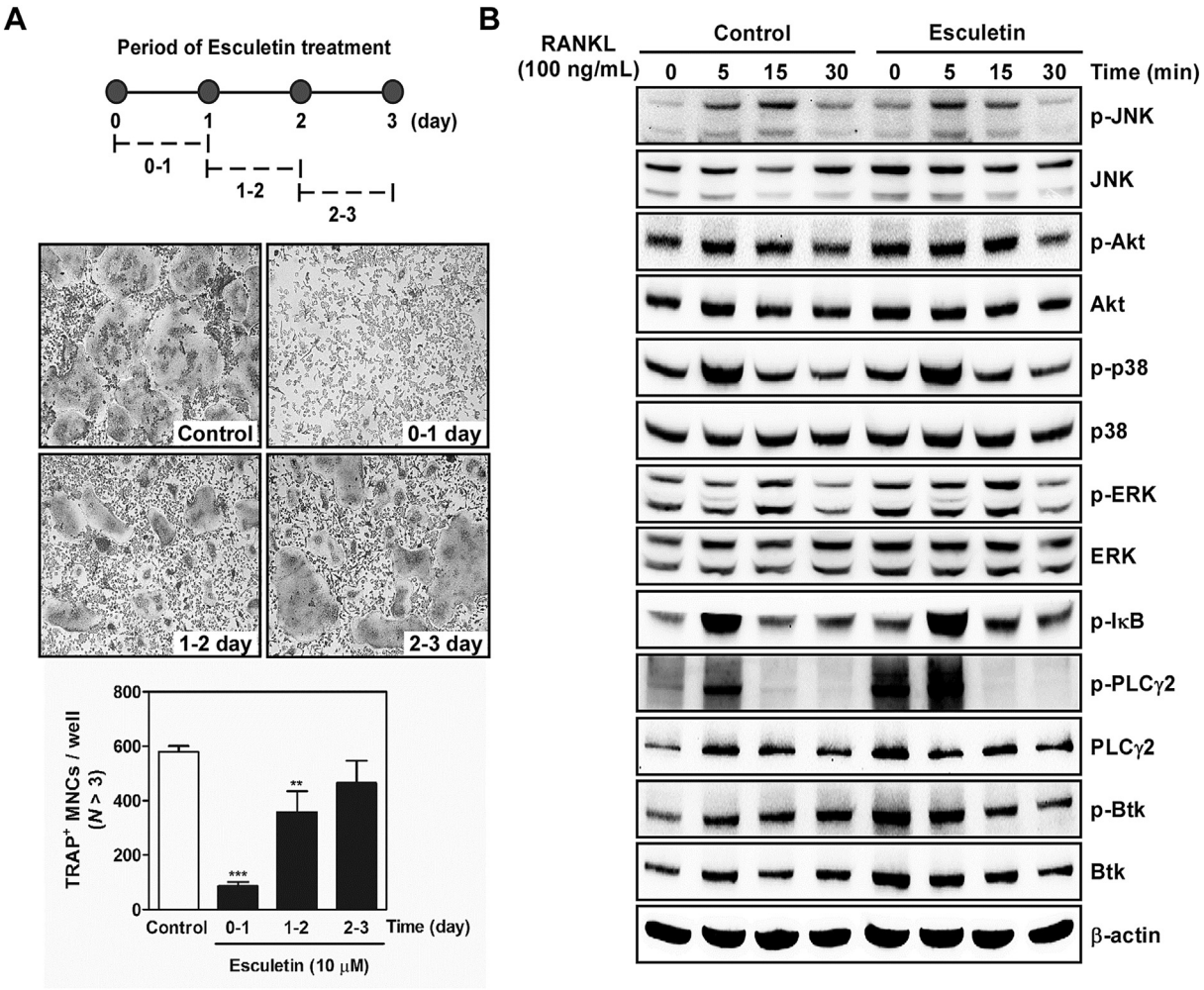

Fig. 2. Effect of esculetin on RANKL-induced signaling pathway (A) BMMs were cultured for 3 days in the presence of M-CSF (30 ng/mL) and RANKL (100 ng/mL) with or without esculetin (10 μM) for the indicated times. The cells were stained with TRAP solution. Diagram of the treatment schedule is presented above TRAP staining data. $TRAP^+$ MNCs with more than three nuclei were counted as osteoclasts. $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* control group. (B) BMMs were pretreated with or without of esculetin (10 μM) for 1 h in the presence of M-CSF (30 ng/mL) before RANKL (100 ng/mL) stimulation at the indicated time points. Whole-cell lysates were analyzed by western blotting with the indicated antibodies. β-actin was used as the internal control.

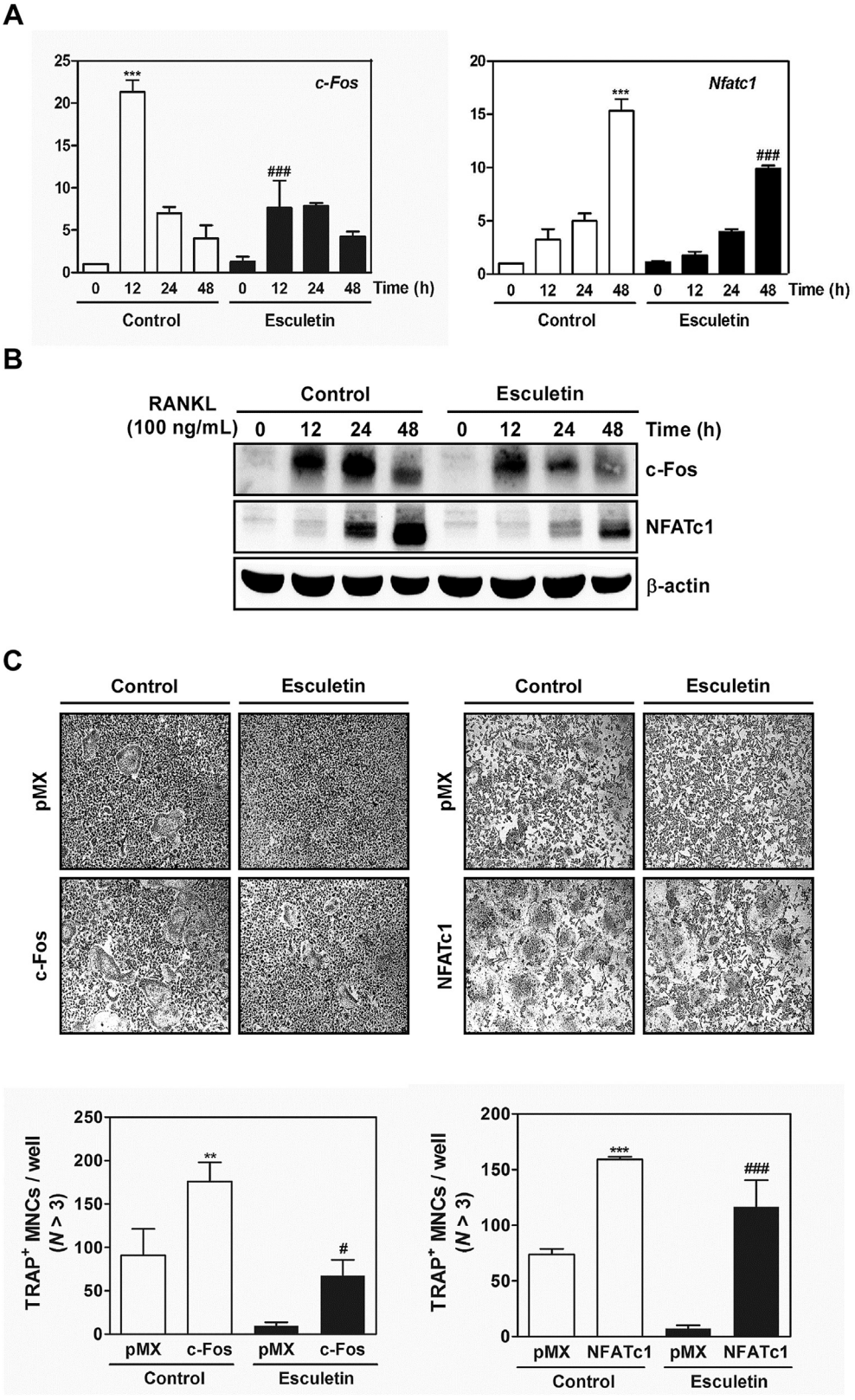

Fig. 3. Esculetin suppresses RANKL-induced c-Fos and NFATc1 expression (A) BMMs were pretreated with or without esculetin (10 μM) for 1 h in the presence of M-CSF (30 ng/mL) and then stimulated with RANKL (100 ng/mL) for the indicated times. The mRNA expression levels of *c-Fos* and *Nfatc1* were analyzed by quantitative real-time RT-PCR. $^{***}P < 0.001$ *vs.* control group at 0 h and $^{###}P < 0.001$ *vs.* control group at the indicated time. (B) Effects of esculetin on c-Fos and NFATc1 protein levels were evaluated by western blot analysis with the indicated antibodies. β-actin was used as the internal control. (C) BMMs were infected with retroviruses expressing pMX, c-Fos, and NFATc1. Infected BMMs were cultured with or without esculetin (10 μM) in the presence of M-CSF (30 ng/mL) and RANKL (50 ng/mL) for 4 days. After culture, the cells were stained with TRAP solution. TRAP-positive cells were photographed under a light microscope. $TRAP^+$ MNCs with more than three nuclei were counted as osteoclasts. $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* control group and $^{#}P < 0.05$, $^{###}P < 0.001$ *vs.* esculetin group.

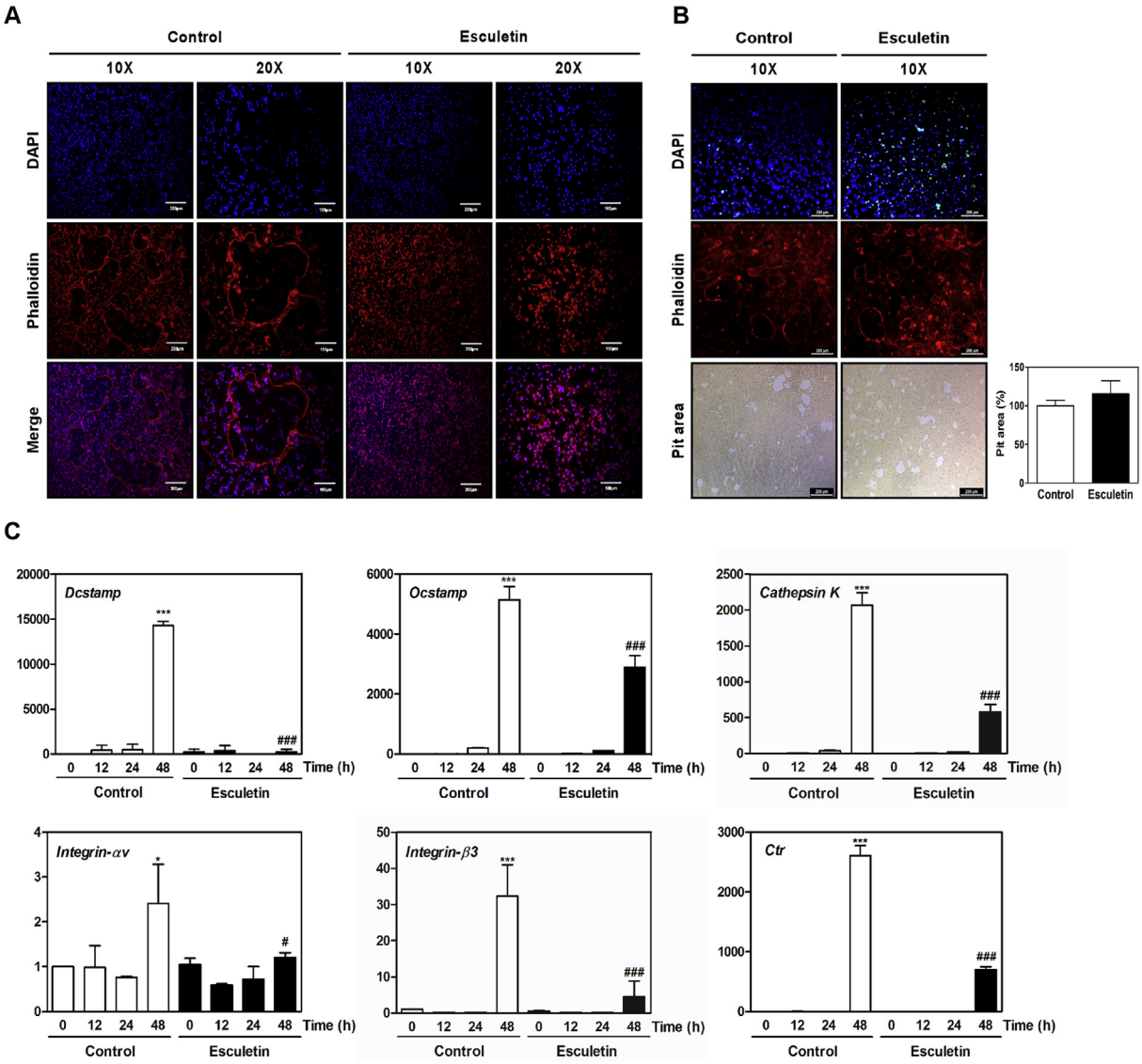

Fig. 4. Effect of esculetin on actin ring formation and bone resorbing activity of mature osteoclasts (A) BMMs were cultured for 4 days with M-CSF (30 ng/mL) and RANKL (100 ng/mL) in the absence or presence of esculetin (10 μM). The cells were fixed, permeabilized, and stained with phalloidin and DAPI and then examined with a confocal laser scanning microscope. (B) Mature osteoclasts were seeded on hydroxyapatite-coated plates and treated with esculetin (10 μM) for 24 h. Attached cells on the plates were stained with phalloidin and DAPI. F-actin ring distribution was visualized using a fluorescence microscope. Next, the attached cells were removed and the plates were photographed under a light microscope. Pit areas on hydroxyapatite plates were quantified using the Image Pro-PLUS (Ver. 4.5) software. (C) BMMs were pretreated with or without esculetin (10 μM) for 1 h in the presence of M-CSF (30 ng/mL) and then stimulated with RANKL (100 ng/mL) for the indicated times. Total RNA isolated from cells using QIAzol reagent and mRNA expression levels of *Dcstamp*, *Ocstamp*, *cathepsin K*, *integrin-αv*, *integrin-β3*, and *Ctr* were evaluated by quantitative real-time RT-PCR. $^{*}P < 0.05$, $^{***}P < 0.001$ *vs.* control group at 0 h and $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ *vs.* control group at the indicated time, respectively. Scale bar = 100 μm or 200 μm.

tested the effects of esculetin on the expression of c-Fos and NFATc1 both at the mRNA and protein level. When BMMs were stimulated with RANKL for the indicated time points, *c-Fos* and *Nfatc1* mRNA expression was upregulated in the control group and significantly reduced by esculetin (Fig. 3A). Similarly, esculetin suppressed c-Fos and NFATc1 protein expression (Fig. 3B). Next, to confirm the possibility that the expression of c-Fos or NFATc1 is sufficient to reverse the effects of esculetin on osteoclastogenesis, we applied a retrovirus overexpressing c-Fos or NFATc1. As shown in Fig. 3C, c-Fos or NFATc1 infection rescued the inhibitory effect of esculetin on osteoclast differentiation. These results suggest that esculetin efficiently inhibits the transcription of *c-Fos and Nfatc1* during RANKL-mediated osteoclastogenesis.

3.4. Esculetin abolishes actin ring formation during osteoclastogenesis, while it has no effects on both F-actin structure and bone resorption of mature osteoclasts

We cultured BMMs with or without esculetin to examine the effect of esculetin on actin ring formation. Esculetin blocked the F-actin structure during BMM differentiation (Fig. 4A). Additionally, to investigate whether esculetin affects the bone-resorbing activity of mature osteoclasts produced in a co-culture system, the cells were treated with or without esculetin and seeded on top of hydroxyapatite-coated plates. Similar resorption pits were observed in both the control and esculetin groups. In addition, F-actin ring of mature osteoclasts was not influenced by esculetin (Fig. 4B). Next, we examined the effects of esculetin on the mRNA

expression of various transcriptional factors, including *Dcstamp*, *Ocstamp*, *cathepsin K*, *integrin-αv*, *integrin-β3*, and *Ctr*, which are closely associated with osteoclast formation and function. *Dcstamp*, *Ocstamp*, *cathepsin K*, *integrin-αv*, *integrin-β3*, and *Ctr* mRNA expression was significantly inhibited by esculetin (Fig. 4C).

4. Discussion

Our study demonstrated that esculetin suppresses RANKL-mediated osteoclast formation. We showed that esculetin effectively attenuated the differentiation of primary BMMs into osteoclast-like TRAP-positive cells. Additionally, the intracellular mechanisms involved in the anti-osteoclastic activity of esculetin was assessed both at the mRNA and protein levels. Esculetin directly induced the downregulation of osteoclast-related transcriptional factors, c-Fos and NFATc1, without influencing RANKL-dependent early signaling pathways, subsequently bringing about the downregulation of downstream osteoclast-related NFATc1 target genes.

c-Fos belongs to the Fos gene family, including *c-Fos*, *FosB*, *Fra-1*, and *Fra-2*, which is a component of the activator protein-1 (AP-1) family of transcriptional activators. c-Fos essentially heterodimerizes with Jun proteins such as c-Jun, JunB, and JunD to accomplish the cooperative DNA sequence binding activity of AP-1. Additionally, AP-1 expression is closely associated with RANKL signaling in osteoclastogenesis *via* interaction of c-Fos with its transcriptional target, fos-like antigen 1 (Fosl1) [21]. A previous study revealed that murine monocyte precursor cells lacking c-Fos have a tendency to differentiate into macrophages rather than osteoclasts *in vitro* and mice lacking endogenous c-Fos suffer from osteopetrosis *in vivo*, suggesting a critical role of c-Fos in osteoclast determination [22]. Another key regulator of osteoclast development is NFATc1, a member of the NFAT transcription factor family in the hematopoietic system, including NFATc1 (NFAT2), NFATc2 (NFAT1), NFATc3 (NFAT4), NFATc4 (NFAT3), and NFAT5. A previous report indicates that NFATc1 induction and activation is strongly induced in response to RANKL stimulus, integrating RANKL-evoked intracellular signaling cascades for the differentiation of osteoclasts. Viable mice with genetic ablation of *Nfatc1* are unable to form mature osteoclasts and undergo subsequent osteopetrotic defect. Moreover, normal osteoclast differentiation is not achieved in a culture system of NFATc1-deficient embryonic stem (ES) cells, whereas NFATc1 ectopic expression efficiently allows precursor cells to differentiate into functional osteoclasts even in the absence of RANKL stimulus [14,23]. In this study, we demonstrated that c-Fos and NFATc1 activation is regulated by esculetin and that this molecular mechanism underlies esculetin's inhibitory action on RANKL-induced osteoclast differentiation (Fig. 3).

At the late stage of osteoclast differentiation, formation of actin-rich structures with a dense F-actin core is required for the attachment to extracellular bone matrix and subsequent bone destruction. Functional osteoclasts form the hallmark of bone resorption, a sealing zone within the resorption lacunae, in which hydrogen ions such as hydrochloric acid and proteolytic enzymes are secreted *via* interaction with αvβ3 integrin, resulting in the dissolution of mineral hydroxyapatite and degradation of the organic matrix [24–28]. As shown in Fig. 4, actin filament assembly was abolished during the differentiation of BMMs, while the inhibitory action of esculetin on the structural organization of F-actin along with bone resorption was not observed in mature osteoclasts. This result indicates that esculetin acts as a negative regulator of actin ring formation through targeting osteoclast precursor cells rather than mature multinucleated osteoclasts.

In summary, our data suggests that esculetin attenuates RANKL-mediated osteoclast differentiation from primary BMMs *via* the suppression of c-Fos and NFATc1 expression independently of RANKL-dependent early signal pathways. Esculetin interferes with the formation of F-actin structure during osteoclastogenesis, while esculetin does not exert this blocking effect on mature osteoclasts. Although further experiments are needed to determine the bone-restoration effects of esculetin *in vivo* in order to propose esculetin as a potential therapeutic agent in the treatment of bone disorders such as osteoporosis, this is the first report to show the anti-osteoclastogenic action of esculetin.

Conflict of interest

The authors declare no competing financial interest.

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Transparency document

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